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1. Your reference — PA 445

2. Patent application number *(The Patent Office will fill in this part)* — **9824632.5**

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CELLTECH THERAPEUTICS LIMITED
216 BATH ROAD
SLOUGH
SL1 4EN

Patents ADP number *(if you know it)* —

If the applicant is a corporate body, give the country/state of its incorporation — UK GC725770C1.

4. Title of the invention

BIOLOGICAL COMPOUNDS

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BIOLOGICAL COMPOUNDS

This invention relates to modified antibody fragments, to processes for their preparation and to their use in medicine.

Since antibodies show great specificity in their binding to other molecules, they are of benefit as therapeutic or diagnostic agents, or as reagents (for instance, as affinity purification reagents or as catalytic enzymes). The advent of hybridoma technology and the generation of transgenic animals have allowed the production of large volumes of monoclonal antibodies, sufficient for therapeutic use. Most such antibodies have been generated for treatment of acute diseases, such as particular types of cancer. For the treatment of chronic and/or more common diseases, still larger amounts of antibody are likely to be required. An increased usage also brings an increased need for a cheaper therapeutic agent.

Monoclonal antibodies can be produced in cultured mammalian or insect cells, or in transgenic animals, but the ability of these technologies to supply a large market such as for treatment of a chronic disease, at a reasonable cost, is uncertain. Microbial systems (bacteria or fungi) offer a potentially larger and cheaper production source. For instance, the bacterium *Escherichia coli* is capable of producing fragments of antibody, such as Fab [Better, M., *et al* (1988) Science, 240, 1041-1043]. However, while antibody fragments are useful for some purposes, they can lack various of the characteristics of whole immunoglobulin molecule. These other characteristics are associated with parts of the immunoglobulin molecule (in the Fc region) that are remote from that which binds the antigenic epitope (the Fab), and so are frequently not present on antigen-binding antibody fragments. Thus, Fab or $F(ab')_2$ fragments of IgG do not show the extended lifetime that is characteristic of the whole IgG molecule because they lack the site on the Fc domain that is responsible for binding to the FcRn receptor that recycles antibody molecules and imparts a long lifetime *in vivo* [Medesan, C., *et al* (1997) J. Immunol. 158, 2211-2217].

No prokaryote system is known to be capable of producing a whole, functioning antibody in a yield sufficient to give an economic process for large-scale manufacture. Fungi are eukaryotes and have been shown to be able to produce heterologous proteins [e.g. Sleep, D., *et al* (1991) Bio/Technology, 9, 183-187], but expression of whole immunoglobulin G (IgG1) in a fungus (*Saccharomyces cerevisiae*) has been reported to occur only at a low level that would be unattractive as a commercial manufacturing process [Horwitz, A. H., *et al* (1988) Proc. Natl. Acad. Sci. USA, 85, 8678-8682]. Furthermore, glycosylation of proteins expressed in microbial systems is absent or unlike that in mammalian systems, and this may potentially present problems if the protein is used *in vivo*, of immunogenicity and abnormal function (complement activation, binding to Fc receptors, transcytosis and prolongation of half-life through interaction with FcRn receptor) [Nose, M. and Wigzell, H. (1983) Proc. Natl. Acad. Sci. USA, 80, 6632-6636; Tao, M.-H. and Morrison, S. L. (1989) J. Immunol. 143, 2595-2601; Wawrzynczak, E. J., *et al* (1992) Molec. Immunol. 29, 213-220; Kim, J.-K., *et al* (1994) Eur. J. Immunol. 24, 2429-2434].

A therapeutic or diagnostic antibody for use *in vivo* would typically be of the G class (IgG). The persistence of IgG in circulation (and in extravascular compartments) is dependent, in detail, on the IgG subclass, as well as the species under discussion. In mice, rats or rabbits the catabolic rate is relatively high - whole IgG β-phase half-life (the time taken for half of the molecules in circulation to be catabolised) is of the order of 4 to 9 days [Waldmann, T. A. and Strober, W. (1969) Progr. Allergy, 13, 1-110; Vieira, P. and Rajewsky, K. (1988) Eur. J. Immunol. 18, 3132-316; Yasmeen, D., *et al* (1976) J. Immunol. 116, 518-526]. In man, however, IgG is catabolised more slowly, with a β-phase half-life of about 20 days, except for IgG3 which is catabolised at about twice that rate [Waldmann, T. A. and Strober, W. (1969) Progr. Allergy, 13, 1-110].

The β-phase half-lives of IgG fragments that can be made in bacterial systems, such as Fab' or $F(ab')_2$, are very much shorter than for the corresponding whole molecule, being reported as only five hours or less

[as reviewed by Waldmann, T. A. and Strober, W. (1969) Progr. Allergy, 13, 1-110]. Again, Kitamura *et al* [Cancer Res. (1991) 51, 4310-4315] measured α- and β-phases of a murine $F(ab')_2$ in mice to be 3.6h and 26.7h respectively. Thus, while they may be cheap to produce, these rapidly-eliminated fragments are of limited therapeutic use.

This has led to attempts to prolong half-life, for instance by modifying the Fab' or $F(ab')_2$ *in vitro* by addition of one or more molecules of polyethylene glycol to each fragment molecule. For example, addition of polyethylene glycol to a $F(ab')_2$ increased its α-phase half-life from 3.6 to 5.6h and its β-phase half-life from 26.7 to 33.6h [Kitamura, K., *et al* (1991) Cancer Res. 51, 4310-4315]. The mechanisms underlying this possibly include reduction in elimination by filtration at the kidney (due to a significant increase in molecular weight), and protection of the protein from degradation by proteases. Unfortunately, this approach entails extra production cost in the form of extra materials and resource, together with reduction in overall yield of the product. There may also be a reduction in binding by antigen, due to interference by the randomly-attached polyethylene gycol [for instance, see Delgado, C., *et al* (1996) Brit. J. Cancer 73, 175-182].

There is, therefore, an identifiable need for a means to economically produce an IgG fragment that can bind antigen and that has a long half-life *in vivo*. To be able to extend the half-life to that of a whole IgG molecule, or to even longer times, would be an additional advantage.

A variety of proteins exist in plasma, circulating in the body with half-lives measured in days, for example, 5 days for thyroxine-binding protein or 2 days for transthyretin [Bartalena, L. and Robbins, J. (1993) Clinics in Lab. Med. 13, 583-598], or 65 h in the second phase of turnover of iodinated α1-acid glycoprotein [Bree, F. *et al* (1986) Clin. Pharmacokin. 11, 336-342]. Again, data from Gitlin et al. [Gitlin, D., *et al* (1964) J. Clin. Invest. 10, 1938-1951] suggest a half-life of 3.8 days for α1-acid glycoprotein in pregnant women, 12 days for transferrin and 2.5 days for fibrinogen.

An example of a protein that has an even longer *in vivo* half-life is serum albumin (hereinafter called albumin), an abundant protein in both vascular and extravascular compartments [see review by Peters, Jr., T. (1985) Adv. Prot. Chem. 37, 161-245]. The half-life of albumin in man is about 19 days (Peters,1985 *ibid*), though it is less in other species - about 2 days in rats (Peters 1985 *ibid*), or about 5 days in rabbits [Hatton, M. W. C., *et al* (1993) J. theor. Biol. 161, 481-490]. In addition, recombinant albumin has been reported as being produced at several gm per litre of culture of yeast (*Pichia pastoris* or *Kluyveromyces lactis* [Barr, K. A., *et al* (1992) Pharm. Eng. 12, 48-51; Fleer, R., *et al* (1991) Bio/Technology 9, 968-975; Cregg, J. M., *et al* (1993) Bio/Technology 11, 905-910]). This level of expression makes industrial production of pharmaceutical grade protein economically feasible, as remarked by Fleer *et al* [Fleer, R., *et al* (1991) Bio/Technology 9, 968-975]. Similarly, transgenic mice have been found to express as much as 10 gm per litre of albumin in their milk [Hurwitz, D. R, *et al* (1994) Transgenic Res. 3,365-375].

Albumin has the function of transportation of various molecules (such as steroid hormones, bile pigments and fatty acids) in circulation and in extravascular fluids, but does not possess the noted ability of antibodies to specifically bind ligands, particularly those of high molecular weight. As with various other serum proteins, albumin is transported poorly across the placenta: labelled albumin injected into a mother appears with 5% or less specific activity in the foetus after 25 days [Gitlin, D., *et al* (1964) J. Clin. Invest. 10, 1938-1951]. This contrasts with IgG, which is transported efficiently across the placenta to the foetus. Similarly, albumin is not transported efficiently across the gut wall of neonates, whereas IgG is, by interaction with the FcRn receptor.

We have now produced a series of hybrid proteins, namely a combination of a serum carrier protein and an antibody fragment - in order to bring together in one molecule, the desirable characteristics of both components. For example, the hybrids can be expected to have the antigen-binding capabilities of the antibody and the longevity of the carrier protein *in vivo*. In addition, hybrids which lack the Fc region of IgG would have significantly reduced access to a foetus since, like albumin

and unlike IgG, they have no transport mechanism to carry them through the placenta. Advantageously, this would give protection to the developing foetus from the possibly detrimental effects of antibody (e.g. cytokine-neutralising activity) administered to the mother. By the same mechanism (lack of Fc), hybrid molecule administered to a mother and so present in her milk would not be taken up by neonate(s) receiving the milk, and so protection to the baby is afforded here, also.

Thus according to one aspect of the invention we provide a multi-component hybrid protein comprising one or more antigen-binding antibody fragments covalently linked to one or more serum carrier proteins or fragments thereof.

Depending on the intended specific use and/or half-life required the hybrid protein according to the invention may be in a number of different forms. For example, one protein according to the invention may comprise an antibody fragment covalently linked to two, three or more serum carrier proteins or fragments thereof each of which may be the same or different. In another example, a protein according to the invention may comprise two, three or more antibody fragments, which may be the same or different, each covalently linked to the same serum protein or a fragment thereof. In general, however, a preferred hybrid protein according to the invention comprises an antigen-binding antbody fragment covalently linked to one serum carrier portein or a fragment thereof.

Each antigen-binding antibody fragment component in the proteins according to the invention will in general comprise an antibody variable region domain containing one or more antigen binding sites.

The variable region domain may be of any size or amino acid composition and will generally comprise at least one hypervariable amino acid sequence responsible for antigen binding embedded in a framework sequence. In general terms the variable (V) region domain may be any suitable arrangement of immunoglobulin heavy (V_H) and/or light (V_L) chain variable domains. Thus for example the V region domain

may be monomeric and be a V_H or V_L domain where these are capable of independently binding antigen with acceptable affinity. Alternatively the V region domain may be dimeric and contain V_H-V_H, V_H-V_L, or V_L-V_L, dimers in which the V_H and V_L chains are non-covalently associated. Where desired, however, the chains may be covalently coupled either directly, for example via a disulphide bond between the two variable domains, or through a linker, for example a peptide linker, to form a single chain domain.

The variable region domain may be any naturally occurring variable domain or an engineered version thereof. By engineered version is meant a variable region domain which has been created using recombinant DNA engineering techniques. Such engineered versions include those created for example from natural antibody variable regions by insertions, deletions or changes in or to the amino acid sequences of the natural antibodies. Particular examples of this type include those engineered variable region domains containing at least one CDR and optionally one or more framework amino acids from one antibody and the remainder of the variable region domain from a second antibody.

The variable region domain will in general be capable of selectively binding to an antigen. The antigen may be any cell-associated antigen, for example a cell surface antigen such as a T-cell, endothelial cell or tumour cell marker, or it may be a soluble antigen. Particular examples of cell surface antigens include adhesion molecules, for example integrins such as β1 integrins, e.g. VLA-4, E-selectin, P-selectin or L-selectin, CD2, CD3, CD4, CD5, CD7, CD8, CD11a, CD11b, CD18, CD19, CD20, CD23, CD25, CD33, CD38, CD40, CD45, CDW52, CD69, carcinoembryonic antigen (CEA), human milk fat globulin (HMFG1 and 2), MHC Class I and MHC Class II antigens, and VEGF, and where appropriate, receptors thereof. Soluble antigens include interleukins such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8 or IL-12, viral antigens, for example respiratory syncytial virus or cytomegalovirus antigens, immunoglobulins, such as IgE, interferons such as interferon-α, interferon-β or interferon-γ, tumour necrosis factor-α, tumour necrosis factor-β, colony stimulating factors

such as G-CSF or GM-CSF, and platelet derived growth factors such as PDGF-α, and PDGF-β and where appropriate receptors thereof.

In practice it is generally preferable that the variable region domain is covalently attached at a C-terminal amino acid to at least one other antibody domain or a fragment thereof. Thus, for example where a V_H domain is present in the variable region domain this may be linked to an immunoglobulin C_H1 domain or a fragment thereof. Similarly a V_L domain may be linked to a C_K domain or a fragment thereof. In this way for example the fragment according to the invention may be a Fab fragment wherein the antigen binding domain contains associated V_H and V_L domains covalently linked at their C-termini to a CH1 and C_K domain respectively. The CH1 domain may be extended with further amino acids, for example to provide a hinge region domain as found in a Fab' fragment, or to provide further domains, such as antibody CH2 and CH3 domains.

Each serum carrier protein component in the hybrid proteins according to the invention may be a naturally occurring serum carrier protein or a fragment thereof. Particular examples include thyroxine-binding protein, transthyretin, α1-acid glycoprotein, transferrin, fibrinogen and, especially, albumin, together with fragments thereof. The carrier proteins will in particular be of human origin. Where desired each may have one or more additional or different amino acids to the naturally occcurring sequence providing always that the resulting sequence is functionally equivalent. Fragments include any smaller part of the parent protein which retains the carrier function of the mature sequence.

The antibody and carrier protein components in the hybrid proteins according to the invention may be directly or indirectly covalently linked. Indirect covalent linkage is intended to mean that an amino-acid in an antibody fragment is attached to an amino-acid in a carrier protein through an intervening chemical sequence, for example a spacing or bridging group. Particular spacing or bridging groups include for example aliphatic, including peptide, chains as more particularly described hereinafter. Direct covalent linkage is intended to mean that

an amino-acid in an antibody fragment is immediately attached to an amino-acid in a carrier protein without an intervening spacing or bridging group. Particular examples include disulphide (-S-S-] and amide [-CONH-] linkages, for example when a cysteine residue in one component is linked to a cysteine residue in another through the thiol group in each, and when the C-terminal acid function of one component is linked to the N-terminal amine of the other.

Particular spacing or bridging groups useful to indirectly link an antibody to a carrier protein include optionally substituted aliphatic, cycloaliphatic, heteroaliphatic, heterocycloaliphatic, aromatic and heteroaromatic groups. Particular groups include optionally substituted straight or branched C_{1-20}alkylene, C_{2-20}alkenylene or C_{2-20}alkynylene chains optionally interrupted and/or terminally substituted by one or more -O- or -S- atoms, or $-N(R^1)-$ [where R^1 is a hydrogen atom or a C_{1-6}alkyl group], $-N(R^1)CO-$, $-CON(R^1)-$, $-N(R^1)SO_2-$, $-SO_2N(R^1)-$, -C(O)-, -C(O)O-, -OC(O)-, -S(O)-, $-S(O)_2-$, aromatic, e.g. phenyl, hereroaromatic, e.g. pyridyl, or cycloalkyl, e.g. cyclohexyl groups. Such chains include for example optionally substituted straight or branched C_{1-10}alkylene chains such as optionally substituted butylene, pentylene, hexylene or heptylene chains, single amino acid residues and peptide chains, for example containing two to twenty amino acids, which may be the same or different, e.g. polyglycine chains such as $(Gly)_n$ where n is an integer from two to ten. Optional substituents which may be present on any of the above chains include one or more halogen atoms, e.g. fluorine, chlorine, bromine or iodine atoms, or C_{1-6}alkyl, C_{1-6}alkoxy, C_{1-6}haloalkyl, C_{1-6}haloalkoxy, -OH or $-N(R^1)(R^2)$ [where R^2 is as defined for R^1] groups.

Where a spacing or bridging group indirectly links an antibody to a carrier porein the linkage may be to the side chain of any suitable amino acid, for example a lysine, arginine, serine, aspartic acid, glutamic acid or cysteine residue, located in the antibody or carrier protein. At each point of attachment the residue of a reactive group may be present. For example, where the spacing or bridging group is linked to a cysteine residue in the antibody or carried protein, the residue of a thiol-selective

reactive group such as a maleimide group or the like may be incorporated as part of the attachment.

Where desired, the hybrid protein according to the invention may have one or more effector or reporter molecules attached to it and the invention extends to such modified proteins. The effector or reporter molecules may be attached to the antibody fragment and/or the carrier protein through any available amino acid side-chain or terminal amino acid functional group located in either component, for example any free amino, imino, hydroxyl or carboxyl group. The linkage may be direct or indirect, through spacing or bridging groups, as just described above, for linking the antibody and carrier protein components.

Effector molecules include, for example, antineoplastic agents, toxins (such as enzymatically active toxins of bacterial or plant origin and fragments thereof e.g. ricin and fragments thereof) biologically active proteins, for example enzymes, nucleic acids and fragments thereof, e.g. DNA, RNA and fragments thereof, radionuclides, particularly radioiodide, and chelated metals. Suitable reporter groups include chelated metals, fluorescent compounds or compounds which may be detected by NMR or ESR spectroscopy.

Particular antineoplastic agents include cytotoxic and cytostatic agents, for example alkylating agents, such as nitrogen mustards (e.g. chlorambucil, melphalan, mechlorethamine, cyclophosphamide, or uracil mustard) and derivatives thereof, triethylenephosphoramide, triethylenethiophosphor-amide, busulphan, or cisplatin; antimetabolites, such as methotrexate, fluorouracil, floxuridine, cytarabine, mercaptopurine, thioguanine, fluoroacetic acid or fluorocitric acid, antibiotics, such as bleomycins (e.g. bleomycin sulphate), doxorubicin, daunorubicin, mitomycins (e.g. mitomycin C), actinomycins (e.g. dactinomycin) plicamycin, calichaemicin and derivatives thereof, or esperamicin and derivatives thereof; mitotic inhibitors, such as etoposide, vincristine or vinblastine and derivatives thereof; alkaloids, such as ellipticine; polyols such as taxicin-I or taxicin-II; hormones, such as androgens (e.g. dromostanolone or testolactone), progestins (e.g.

megestrol acetate or medroxyprogesterone acetate), estrogens (e.g. dimethylstilbestrol diphosphate, polyestradiol phosphate or estramustine phosphate) or antiestrogens (e.g. tamoxifen); anthraquinones, such as mitoxantrone, ureas, such as hydroxyurea; hydrazines, such as procarbazine; or imidazoles, such as dacarbazine.

Particularly useful effector groups are calichaemicin and derivatives thereof (see for example South African Patent Specifications Nos. 85/8794, 88/8127 and 90/2839).

Chelated metals include chelates of di-or tripositive metals having a coordination number from 2 to 8 inclusive. Particular examples of such metals include technetium (Tc), rhenium (Re), cobalt (Co), copper (Cu), gold (Au), silver (Ag), lead (Pb), bismuth (Bi), indium (In), gallium (Ga), yttrium (Y), terbium (Tb), gadolinium (Gd), and scandium (Sc). In general the metal is preferably a radionuclide. Particular radionuclides include ^{99m}Tc, ^{186}Re, ^{188}Re, ^{58}Co, ^{60}Co, ^{67}Cu, ^{195}Au, ^{199}Au, ^{110}Ag, ^{203}Pb, ^{206}Bi, ^{207}Bi, ^{111}In, ^{67}Ga, ^{68}Ga, ^{88}Y, ^{90}Y, ^{160}Tb, ^{153}Gd and ^{47}Sc.

The chelated metal may be for example one of the above types of metal chelated with any suitable polydentate chelating agent, for example acyclic or cyclic polyamines, polyethers, (e.g. crown ethers and derivatives thereof); polyamides; porphyrins; and carbocyclic derivatives.

In general, the type of chelating agent will depend on the metal in use. One particularly useful group of chelating agents in conjugates according to the invention, however, are acyclic and cyclic polyamines, especially polyaminocarboxylic acids, for example diethylenetriaminepentaacetic acid and derivatives thereof, and macrocyclic amines, e.g. cyclic tri-aza and tetra-aza derivatives (for example as described in International Patent Specification No. WO 92/22583); and polyamides, especially desferrioxamine and derivatives thereof.

Particularly useful hybrid proteins according to the invention include one or more antigen-binding antbody fragments covalently linked to one or more albumin molecules or fragments thereof.

Particular hybrids of this type include those wherein one antigen-binding antibody fragment is covalently linked to an albumin molecule or a fragment thereof. In these hybrids, and in general in the proteins according to the invention, each antigen-binding antibody fragment is preferably a monovalent Fab fragment, optionally containing one or more additional amino acids attached to the C-terminus of the CH1 domain and is especially a Fab' fragment. Particularly useful Fab' fragments include those wherein the hinge domain contains a single cysteine residue. Fragments of albumin include one or more of domains I, II and/or III or subdomains thereof [see for example Peters, T in "All about Albumin", Academic press, London (1996)].

Especially useful antibody-albumin hybrids according to the invention include those in which each protein component is directly linked through the C-terminal amino acid of the antibody to the N-terminal amino acid of the albumin. Where desired, one or more additional amino acids may be inserted between the C- and N-terminii to form a spacing group.

Another particularly useful class of antibody-albumin hybrids according to the invention is that wherein each protein component is indirectly linked between a thiol group of a cysteine residue present in the antibody and in the albumin. The linking group may be a spacing or bridging molecule as described above. Particularly useful groups include non-cleavable linker groups, especially optionally substituted straight or branched C_{1-10}alkylene chains.

In this class of hybrids the antibody is preferably a Fab fragment optionally containing one or more additional amino acids attached to the C-terminal of the CH1 domain. Especially useful fragments include Fab' fragments. The cysteine residue to which the spacing or bridging molecule is attached is preferably located in the CH1 domain of the Fab or, especially, is located in any C-terminal extension of the CH1 domain of the Fab, for example in the hinge domain of a Fab'.

The albumin in this class of hybrids may be in particular mature human serum albumin or a fragment thereof. In this instance, the spacing or briding molecule may be attached to the cysteine residue at position 34. Advantageously, to avoid undesirable homodimer formation [see the Examples below] the spacing or bridging molecule may be from around 10Å to around 20Å in length, for example around 16Å. Suitable bridging molecules in this length range may be easily determined from published sources, for example manufacturers' catalogues [see below]. Particularly useful bridging molecules include optionally substituted hexylene chains. Where each end of the bridging molecule is attached to the cysteine residue this may be through a disulphide bond or, in particular, a sulphur-carbon bond. Where the linkage is a sulphur-carbon bond, the residue of a thiol-selective reactive group, such as a maleimide, may be present as part of each end of the spacing or bridging group.

The hybrid proteins according to the invention may be useful in the detection or treatment of a number of diseases or disorders. Such diseases or disorders may include those described under the general headings of infectious disease, e.g. viral infection; inflammatory disease/autoimmunity e.g. rheumatoid arthritis, osteoarthritis, inflammatory bowel disease; cancer; allergic/atopic disease e.g. asthma, eczema; congenital disease, e.g. cystic fibrosis, sickle cell anaemia; dermatologic disease, e.g. psoriasis; neurologic disease, e.g. multiple sclerosis; transplants e.g. organ transplant rejection, graft-versus-host disease; and metabolic/idiopathic disease e.g. diabetes.

The hybrid protein according to the invention may be formulated for use in therapy and/or diagnosis and according to a further aspect of the invention we provide a pharmaceutical composition comprising a multi-component hybrid protein comprising one or more antigen-binding antibody fragments covalently linked to one or more serum carrier proteins or fragments thereof, together with one or more pharmaceutically acceptable excipients, diluents or carriers.

As explained above, the hybrid protein in this aspect of the invention may be optionally linked to one or more effector or reporter groups.

The pharmaceutical composition may take any suitable form for administration, and, preferably is in a form suitable for parenteral administration e.g. by injection or infusion, for example by bolus injection or continuous infusion. Where the composition is for injection of infusion, it may take the form of a suspension, solution or emulsion in an oily or aqueous vehicle and it may contain formulatory agents such as suspending, preservative, stabilising and/or dispersing agents.

Alternatively, the composition may be in dry form, for reconstitution before use with an appropriate sterile liquid.

If the composition is suitable for oral administration the formulation may contain, in addition to the active ingredient, additives such as: starch e.g. potato, maize or wheat starch or cellulose or starch derivatives such as microcrystalline cellulose; silica; various sugars such as lactose; magnesium carbonate and/or calcium phosphate. It is desirable that, if the formulation is for oral administration it will be well tolerated by the patient's digestive system. To this end, it may be desirable to include in the formulation mucus formers and resins. It may also be desirable to improve tolerance by formulating the antibody in a capsule which is insoluble in the gastric juices. It may also be preferable to include the antibody or composition in a controlled release formulation.

If the composition is suitable for rectal administration the formulation may contain a binding and/or lubricating agent; for example polymeric glycols, gelatins, cocoa-butter or other vegetable waxes or fats.

Therapeutic and diagnostic uses of hybrid proteins according to the invention typically comprise administering an effective amount of the proteint to a human subject. The exact amount to be administered will vary according to the intended use of the protein and on the age, sex and condition of the patient but may typically be varied from about 0.1mg to 1000mg for example from about 1mg to 500mg. The protein may be administered as a single dose or in a continuous manner over a period of time. Doses may be repeated as appropriate. Typical doses may be for

example between 0.1-50mg/kg body weight per single therapeutic dose, particularly between 0.1-20 mg/kg body weight for a single therapeutic dose.

The hybrid proteins according to the invention may be prepared by standard chemical, enzymatic and/or recombinant DNA procedures.

Thus for example the hybrid protein may be prepared by the use of recombinant DNA techniques involving the manipulation and re-expression of DNA encoding antibody variable and/or constant regions and a desired carrier protein or a fragment thereof. Such DNA is known and/or is readily available from DNA libraries including for example phage-antibody libraries [see Chiswell, D J and McCafferty, J. Tibtech. 10, 80-84 (1992)] or where desired can be synthesised. Standard molecular biology and/or chemistry procedures may be used to sequence and manipulate the DNA, for example, to introduce codons to create cysteine residues, to modify, add or delete other amino acids or domains in the antibody and/or carrier protein as desired.

From here, one or more replicable expression vectors containing the DNA may be prepared and used to transform an appropriate cell line, e.g. a non-producing myeloma cell line, such as a mouse NSO line or a bacterial, e.g. *E.coli* line, or, especially, a fungal line, such as a yeast line, e.g. members of the genera *Pichia*, *Saccharomyces*, or *Kluyveromyces* in which production of the antibody will occur. In order to obtain efficient transcription and translation, the DNA sequence in each vector should include appropriate regulatory sequences, particularly a promoter and leader sequence operably linked to the variable domain sequence. Particular methods for producing recombinant proteins in this way are generally well known and routinely used. For example, basic molecular biology procedures are described by Maniatis *et al* [Molecular Cloning, Cold Spring Harbor Laboratory, New York, 1989]; DNA sequencing can be performed as described in Sanger *et al* [PNAS 74, 5463, (1977)] and the Amersham International plc sequencing handbook; and site directed mutagenesis can be carried out according to the method of Kramer *et al* [Nucl. Acids Res. 12, 9441, (1984)] and the

Anglian Biotechnology Ltd handbook. Additionally, there are numerous publications, including patent specifications, detailing techniques suitable for the preparation of proteins by manipulation of DNA, creation of expression vectors and transformation of appropriate cells for example as described in International Patent Specification No. WO86/01533 and European Patent Specification No. 392745.

Chemical synthesis of the hybrid proteins according to the invention may be achieved by coupling appropriately functionalised antibody, carrier protein and, where appropriate, spacing or bridging groups in a predetermined order. Standard chemical coupling techniques may be employed utilising starting materials containing one or more reactive functional groups such as thiols, acids, thioacids, anhydrides, acid halides, esters, imides, aldehydes, ketones, imines and amines. The starting antibody and carrier protein may be readily obtained from natural sources and/or by recombinant DNA techniques as described previously. Suitable spacing or bridging groups, for example in the 10Å-20Å length range as described above, are either commercially available [see for example Pierce & Warriner (UK) Ltd., Chester, UK] or may be obtained by simple functionalisation of known readily available chemical using convention chemistry.

Thus in one general approach, a homo- or heterobifunctional spacing or bridging group may first be coupled to either the antibody or carrier protein and the resulting product coupled as necessary to the remaining component to provide the hybrid protein of the invention. The coupling reactions may be performed using standard conditions for reactions of this type. Thus for example the reaction may be performed in a solvent, for example an organic solvent, an aqueous-organic solvent or, especially, an aqueous solvent at or around ambient gemperature up to around 70°C. Preferably, to avoid unwanted dimerisation in the first coupling reaction the homo- or heterobifunctional spacing or bridging group is employed in excess concentration relative to the antibody or carrier protein. Similarly in the second coupling reaction, the antibody or carrier protein is preferably employed in excess concentration to the product of the first coupling reaction.Illustrative reactions are described in

detail in the Examples hereinafter for the preparation of proteins according to the invention and these may be readily adapted using different starting materials to provide other compounds of the invention.

The following Examples illustrate the invention.

EXAMPLES

Example 1.

Methods

Preparation of anti-TNF Fab'

Recombinant anti-TNF Fab was produced in *E. coli*, and prepared from the periplasm by the methods described in International Patent Specification No. WO98/25971,

Conjugation of the anti-TNF Fab' with rat serum albumin

Rat serum albumin (RSA, fraction V, Sigma, code no. A-6272) was dissolved to 6.7 mg/mL (0.1 mM) in sodium acetate, 0.1M, pH 5.9. Dithiothreitol solution (100 mM in the same acetate buffer) was added to give a final dithiothreitol concentration of 0.3M, so giving a 3-fold molar excess over RSA. The mixture was incubated at 37°C for 40 min. The mixture was then subjected to chromatography on Sephadex G25M using a PD10 column (Pharmacia, code no. 17-0851-01, used as per manufacturer's instructions), thus removing dithiothreitol and exchanging the buffer for sodium phosphate, 0.1M, pH6, 2mM ethylenediamine-tetraacetate (EDTA). The RSA concentration at that stage was 70μM.

1,6 bismaleimidohexane (BMH, Pierce, code no. 22330) was dissolved to 7.736 mg/mL (28 mM) in dimethylformamide. The BMH solution was added to the reduced RSA solution to give a 21-fold molar excess of BMH over RSA. The mixture was incubated at 21°C for 100 min, then subjected to chromatography on G25M in a buffer of sodium phosphate, 0.1 M, pH6, 2mM EDTA. The concentration of derivatised RSA was 46 μM at that stage.

The solutions of derivatised RSA and the anti-TNF Fab' (187μM) in sodium phosphate, 0.1M, pH6, 2mM EDTA were mixed to give a molar

ratio, RSA:Fab'::1:1.3 (which, corrected for derivatisation of RSA and reduction of Fab' thiol gave a ratio, derivatised RSA:reduced Fab'::1:1.4). The mixture was incubated at 21°C for 2h, although reaction was essentially complete within 1h. The mixture was then stored at 4°C until subjected to purification procedures.

Conjugation of Fab' with cysteine

The method for preparation of control molecule, anti-TNF Fab' covalently linked by BMH via thiols to cysteine (instead of RSA) was essentially the same as for preparation of conjugate (see above). 20µM Fab' was reacted at 21°C for 95 min with a 40-fold molar excess of BMH (added as a solution in dimethylformamide). After chromatography on G25M (to remove BMH) the derivatised Fab' was reacted with cysteine (Sigma, code no. C-4820) at a molar ratio Fab':cysteine free thiol::1:4.5. After reaction at 21°C for 160 min, the sample was stored at 4°C prior to purification of the Fab'-cys product.

Purification of the conjugate

The reaction mixture was first subjected to chromatography on Gammabind plus (Pharmacia), a matrix that has affinity for Fab'. A 5.3 mL column was equilibrated in a buffer of sodium phosphate, 0.1M, pH6, 2mM EDTA at a flow rate of 2 mL/min. All chromatography was at a temperature of 21°C. The sample was applied to the column at a flow rate of 1 mL/min, and the column then washed in the same buffer (sodium phosphate, 0.1M, pH6, 2mM EDTA) until the baseline was restored. Adsorbed protein was eluted by application of a buffer of acetic acid, 0.5M, made to pH3 by addition of sodium hydroxide. The whole eluent was collected in fractions and each fraction analysed by SDS PAGE (using both reducing and non-reducing conditions). As expected of this affinity matrix, unconjugated Fab' eluted in the pH3 buffer, whereas the unconjugated RSA did not bind to the matrix at all, and emerged in the flow-through during sample loading. Conjugation of Fab' to RSA clearly affected its binding to the protein G on the matrix, for the conjugate emerged in the flow-through, just slightly later than (and overlapping with) the RSA. The fraction containing the conjugate was subjected once

more to chromatography on Gammabind plus (as above), in order to separate more RSA from it. The fractions containing conjugate (and some traces of RSA) were concentrated in a stirred cell (Amicon, 10 kDa nominal molecular weight cut-off membrane).

The contaminating RSA was removed from the preparation by gel permeation chromatography on a GF250 HPLC column of size 2x23cm (using a Hewlett Packard 1090 HPLC). The column was equilibrated and eluted in a buffer of sodium phosphate, 0.2M, pH7, at a flow rate of 3 mL/min, at 21°C. The sample of concentrated conjugate (and RSA) was chromatographed and elution monitored at 280 and 220nm. Fractions corresponding to observed peaks were collected and analysed by SDS PAGE. Those fractions containing the conjugate (which was completely resolved from the later-eluting RSA) were pooled and concentrated in a stirred cell (Amicon, 10 kDa nominal molecular weight cut-off membrane). The solution was stored at 4°C, with sodium azide added to 0.05% (w/v), to act as a preservative.

Purification of the Fab'-cys

The reaction mixture containing Fab'-cys product was diluted 5-fold in a buffer of sodium acetate, 50mM, pH4.5, then loaded onto a Mono S column (of 1mL volume), using an FPLC (Pharmacia) apparatus. Adsorbed proteins were then eluted in a gradient of 0 to 250mM sodium chloride in sodium acetate, 50mM, pH4.5. Elution (1 mL/min at 21°C) was monitored by absorption at 280nm. Collected peaks were analysed by SDS PAGE.

Radiolabelling of proteins

Proteins were labelled at the ε-amino groups of lysyl residues, using ^{125}I-labeled Bolton and Hunter reagent (Amersham International, code no. IM5861). Proteins were dissolved or diluted in a buffer of borate, to give a final borate concentration of 0.1M, pH8. A solution (of between 300 and 370µL) containing 300µg protein was then mixed with 20µL Bolton and Hunter solution in propan-2-ol (containing 9 MBq of ^{125}I). The mixture was incubated at 21°C for 15 min, then the reaction was quenched by addition of 60µL solution of glycine, 1M, in borate, 0.1M,

pH8.5. After approximately 5 min reaction at 21°C, the reaction mixture was chromatographed on Sephadex G25M using a PD10 column (Pharmacia, code no. 17-0851-01, used as per manufacturer's instructions). In doing so, the buffer was exchanged for phosphate buffered saline. The specific activity of each preparation was calculated from estimates of protein concentration (see Analytical Procedures) and of radioactivity, and were typically in the range 0.45 to 0.54μCi / μg. The radiolabeled samples were used directly after labeling.

Analytical procedures

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS PAGE) utilised precast SDS gels (Novex), 1mm thick and of acrylamide concentration 4 to 20%, run as per manufacturer's instructions. Gels were stained by soaking for 1h in Coomassie BBG in perchloric acid (Sigma, code no. B-8772), followed by washing in water. Various molecular weight standards were used in order to derive approximate molecular weights (apparent) for sample proteins. These standards were Mark 12 and Seeblue unstained and prestained markers, respectively (Novex). For western blots, SDS PAGE was followed by blotting to polyvinylidene difluoride membrane (Millipore), with detection of the Fd fragment of the Fab' by sheep anti-human IgG(Fd) IgG fraction (The Binding Site, code no. PC075) followed by peroxidase-affinipure $F(ab')_2$ fragment of rabbit anti-sheep IgG, Fc fragment (Immunoresearch, code no. 313036046) and visualisation by use of chemiluminescence (ECL; Amersham International). For autoradiography, the SDS PAGE was followed by exposure of the gel to photographic film (Hyperfilm MP; Amersham International). For imaging of radiolabelled samples on gels (and subsequent quantification) the gels were exposed to high resolution or general purpose screens and processed in the Canberra Packard Cyclone system, using Optiquant software.

Quantification of protein solutions was by absorption at wavelength 280nm in a 1cm cell, using absorption coefficients (for 1mg/mL solution in a 1cm cell) of 1.43 for Fab' or $F(ab')_2$, and of 0.58 for RSA. A coefficient of 1.0 for RSA-Fab' conjugate was calculated from those of RSA and

Fab', weighted in accordance with the constituent masses of the two components.

The concentration of free thiol in a protein solution was measured by adding 1/9 volume of 4,4'-dithiodipyridine (5mM, final concentration therefore was 0.5mM) in phosphate buffered saline. After 10min at 21°C, the absorbance at 324nm was measured in a 1cm cell. The absorbance of a buffer-only blank sample was subtracted from this value, and this figure multipled by 56.1167 to give the result in µM thiol (this being further corrected for any dilution of the original sample).

N-terminal protein sequencing was performed as per manufacturer's instructions on a model 470A protein sequencer with on-line 120A HPLC and 900A data analysis system (Applied Biosystems). Protein in solution was adsorbed to polyvinylidene difluoride membrane in a Prosorb device (Applied Biosystems). Proteins from SDS PAGE were blotted to polyvinylidene difluoride membrane (Millipore), and protein bands detected by staining by 0.1% (w/v) Ponceau S (Sigma, code no. P-3504) in 1% (v/v) acetic acid for 1 min, then destaining the background in water. Bands were excised and sequenced directly.

Surface plasmon resonance study of interactions with ligand was performed on a BIACORE 2000 (Biacore AB), as per manufacturer's recommendations. Sensor chip surface coated by goat anti-human $F(ab')_2$ antibody (Jackson ImmunoResearch Lab. Inc.), which binds to the light chain, was used to bind conjugate, Fab' or IgG, whose binding to ligand (TNF) from solution was then measurable.

To assay the neutralisation of TNF, a monolayer of mouse L929 cells was grown in normal RPMI 1640 plus glutamine and 10% (v/v) foetal calf serum. TNF added in presence of actinomycin D (1%, w/v) with or without sample/anti-TNF. Cell death caused by TNF was monitored by MTT assay: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma, code no. M-2128) was added to a final concentration of 50µg/mL in the medium of treated cells and incubated at 37°C for 4h. Reaction was stopped and the brown colour produced by live cells was

solubilised by a solution of SDS, 20% (w/v) in 50% dimethylformamide in water, at pH 4.7 (by addition of 50% acetic acid, 1M, in hydrochloric acid, 1M) and then quantified by measuring absorbance at 570nm. Having subtracted the absorbance at 630nm, the degree of cell survival (and so the concentration of active TNF present) could be assessed by comparison with samples of cells treated by standard amounts of TNF in the absence of TNF-neutralising activity.

Incubation of protein in plasma or blood in vitro

Rat plasma and blood (heparinised) were prepared fresh. To 100µL was added 12µL of ^{125}I-labeled protein solution. Incubation was at 37°C in a capped 0.5mL tube, with 0.5 and 2µL samples withdrawn at intervals far SDS PAGE and autoradiography.

Unlabeled conjugate or Fab'-cys (2µ of 0.9 mg/mL solution) was mixed with 100µL fresh rat plasma and incubated at 37°C in a capped 0.5mL tube. Samples of 2µL were withdrawn at intervals for analysis by western blotting.

Pharmacokinetic analysis of the conjugate

Male Wistar rats (of approximately 250g each) each received 20µg of ^{125}I-labeled, or 180µg of unlabeled protein in solution in phosphate buffered saline, being injected into a tail vein. Samples of blood were taken from the tail artery at intervals thereafter, with plasma prepared by heparinisation and centrifugation. Samples were analysed as described above (see Analytical Procedures) and radioactivity in whole blood was calculated as cpm/g blood. The percent injected dose (%ID) was calculated for each individual rat, based on standards and expressed as %ID / mL total blood volume. Groups of 6 or 2 rats were used for study of ^{125}I-labeled or unlabeled protein, respectively.

Data were analysed by WinNonlin software in order to determine the pharmacokinetic values quoted.

Results

Characterisation of the conjugate

It is known that although serum albumin has one cysteinyl residue that is not engaged in a disulphide bond (residue 34 in the mature human albumin sequence), many of the molecules in a preparation of albumin do not possess a free cysteinyl thiol due to formation of mixed disulphides with molecules such as glutathione or free cysteine (Peters, *ibid* 1985). In accordance with this, analysis of the rat albumin solution prior to reduction showed that there was 0.15 mole of thiol per mole of albumin, i.e. only 15% of albumin molecules possessed a free thiol. After reduction, this was increased to 0.95 mole of thiol per mole of albumin, consistent with production of a free thiol group in 95% of albumin molecules, most likely at position 34 in the mature albumin sequence, since reduction under conditions such as those used here does not disrupt any of the disulphide present elsewhere in the molecule (Peters, *ibid* 1985). Subsequent reaction at this cysteinyl residue allowed attachment of another molecule at a specific site, and in a 1:1 molar ratio, without concomitant production of other products of higher ratio of albumin:other molecule that would need to be removed subsequently. Co-ordinates for the crystal structure of human serum albumin have been deposited in the Brookhaven database (as entry 1AO6, by S. Sugio, S. Mochizuki and A. Kashima). Inspection of this model shows that the free cysteinyl residue at position 34 lies in a groove between two helices, but it is not clear from this how long a spacer arm on a crosslinking agent should be in order to work effectively. Absence of albumin homo-dimer would be advantageous in a production process since the yield of hetero-dimer then would be greater, and also there would be no need for an additional step to remove the homo-dimer. It was found that the cross-linking agent used in the present example produced this advantageous result. Reaction of the reduced albumin with a 20-fold molar excess of BMH caused derivatisation of 80% of these albumin molecules. Albumin homo-dimers were not formed during albumin derivatisation (or subsequent reaction with other protein molecules). The cross-linking agent spacer arm, of length 16.1 Å, was long enough to reach from the cysteinyl thiol at position 34 to the surface of the albumin molecule, where it was able to react with the free thiol on a Fab' molecule. It was not long enough to penetrate the equivalent groove on a second albumin

to reach the free thiol there, and so production of homo-dimers was avoided.

The derivatised albumin and Fab' were mixed in the molar ratio 1:1.4::albumin :Fab'. SDS PAGE analysis (Fig.1) of the products at the end of reaction indicated a yield of albumin-Fab' conjugate of the order of 20 to 30%, though this would be expected to be improved by optimisation of reaction conditions. The conjugate was purified from the reaction mixture by affinity chromatography, the conjugate surprisingly not binding to the matrix as did Fab', but eluting marginally later than did albumin, which did not bind at all. Having removed unreacted Fab', albumin was separated from the conjugate by size exclusion. The final preparation of conjugate was analysed by SDS PAGE [Fig.1 (ii)], which indicated (by apparent molecular weights) the linkage of one Fab' per albumin molecule. Some dimer material was also present, analogous to the observed occurrence of albumin polymers in untreated preparations of albumin. N-terminal protein sequence analysis of the different species seen on SDS PAGE [Fig.2(i)] confirmed the 1:1 stoichiometry of conjugation, in demonstrating N-terminal sequences of rat albumin and Fab' heavy and light chains in equal molar proportions.

Conjugation of Fab' to cysteine (to produce the control molecule, Fab'-cys) gave good yield - virtually all the Fab' derivative reacted with cysteine with only traces of $F(ab')_2$-like material of greater than approximately 55 kDa (apparent) on non-reducing SDS PAGE. This trace of material eluted just after the Fab'-cys product, which eluted at a sodium chloride concentration of about 75mM on Mono S chromatography, and was excluded from it.

The ability to assay the conjugate in an ELISA which depended on the conjugate binding to TNF indicated that the conjugate retained the ability to bind TNF. This was confirmed by analysis of the conjugate binding to TNF by BIACORE (association and dissociation rates, and equilibrium constant - [see Fig. 2(ii)], which showed that the anti-TNF Fab' was unaffected by conjugation to albumin. This was also confirmed by its equal capacity for neutralisation of TNF (in an assay of L929 cell death

caused by TNF the concentration of protein to inhibit 90% of TNF activity, the IC90, for the albumin-anti-TNF Fab' conjugate was 14.7pM, as compared to 18.0pM for anti-TNF Fab'-cysteine conjugate, 10.9pM for anti-TNF $F(ab')_2$, and 11.3 pM for anti-TNF IgG4). This maintenance of Fab' binding activity was attributed to the intended orientation of the Fab' binding domain away from the point of conjugation to the albumin molecule.

The control, Fab'-cys also retained its ability to bind to, and neutralise, TNF, as seen by its detection by ELISA and its activity in the L929 assay.

Incubation of the conjugate in rat plasma (in vitro) at 37°C for 68h in the presence or absence of a variety of protease inhibitors, monitored by SDS PAGE and western blotting, indicated that the conjugate was stable in rat plasma. Conjugate that had been labelled by ^{125}I was incubated *in vitro* in phosphate buffered saline (pH7) for up to 10 days and was also found to be stable. However, incubation of the ^{125}I-labeled conjugate at 37°C in rat plasma or blood *in vitro* indicated that the molecule was not completely stable, some molecules suffering cleavage at or near the point of linkage between the albumin and the Fab' molecules. Despite an apparent instability in these conditions, uncleaved material remained even after 168h incubation. Since unlabeled conjugate was stable, the observed instability was attributed to modification of the protein by the presence of ^{125}I or the labelling process itself. The integrity of the conjugate in vivo was assessed by SDS PAGE of plasma sample, followed by phosphorimager scanning.

Pharmacokinetic analysis of the conjugate in rat plasma

^{125}I-labeled RSA, Fab', $F(ab')_2$ and RSA-Fab' conjugate were monitored in plasma sampled at various times over 144h (Fig.3). The observed β-phase half-life of albumin was in agreement with the literature value of about 2 days (Peters, *ibid* 1985). The β-phase half-life of the $F(ab')_2$, and the Fab', were similar to that of $F(ab')_2$ described previously (e.g. Kitamura *et al, ibid* 1991), and was preceded by rapid elimination in the α-phase, typical of such molecules. However, when the Fab' was conjugated to RSA it persisted in circulation in the rat to a degree

comparable to rat albumin. Due to a reduction of elimination during both α and β- phase phases, the conjugate showed a 35-fold greater area under the curve (AUC) than did the Fab'-cys control, similar to that of albumin alone. In order to ensure that radioactivity detected in the plasma samples reflected remaining conjugate, samples from one rat given labelled conjugate were run on SDS PAGE and scanned by phosphorimager (Fig.4). This showed the persistence *in vivo* of intact labelled conjugate for at least 120h, and the β-phase half-life of intact conjugate was calculated to be 32.07h, in good agreement with the equivalent result from total ^{125}I detection.

The result was further supported by the experiment in which conjugate that was not labelled was monitored by a form of ELISA that only recognised TNF-binding capability linked to albumin, i.e., intact conjugate. Results from use of unlabelled conjugate in just two rats showed again that the conjugate had a greatly reduced α-phase in circulation in the rat, compared to the unlabeled Fab'-cys control, and a β-phase half-life that was in agreement with that observed from ^{125}I-labeled conjugate (Fig.5). The conjugate's AUC was 200 to 300-fold greater than that of the Fab'-cys control. Assay of the Fab' portion of the conjugate (instead of the whole molecule, including the albumin part) gave the same result.

The plasma samples were assayed for TNF neutralising ability, in the L929 assay, and while both Fab'-cys control and conjugate plasma samples retained TNF-neutralising power, that of the latter persisted for days, as opposed to hours for the former (Fig.6). This echoed the results obtained by ELISA, and demonstrated the conjugate's greatly protracted biological activity *in vivo*.

It has been found that it is possible to prepare a Fab' fragment of an anti-cytokine monoclonal IgG, chemically crosslink it to rat albumin *in vitro*, and thereafter show that the conjugate has retained full cytokine-binding ability (having the same affinity as seen in the whole antibody) and that it has a significantly longer half-life *in vivo* than does the unconjugated Fab'. The conjugate contained one albumin per Fab'. The β-phase half-

life of the conjugate in the rat was about twice that of the unconjugated Fab, being closer to that of unconjugated albumin, and the area under the curve was of the order of 200 to 300-fold greater for the conjugate than for the unconjugated Fab'. Thus, the consequence of conjugation of Fab' to albumin was a significant prolongation of its biological activity *in vivo*, allowing (in the present case) prolonged anti-cytokine therapy.

Conjugation of rat serum albumin and Fab' by BMH

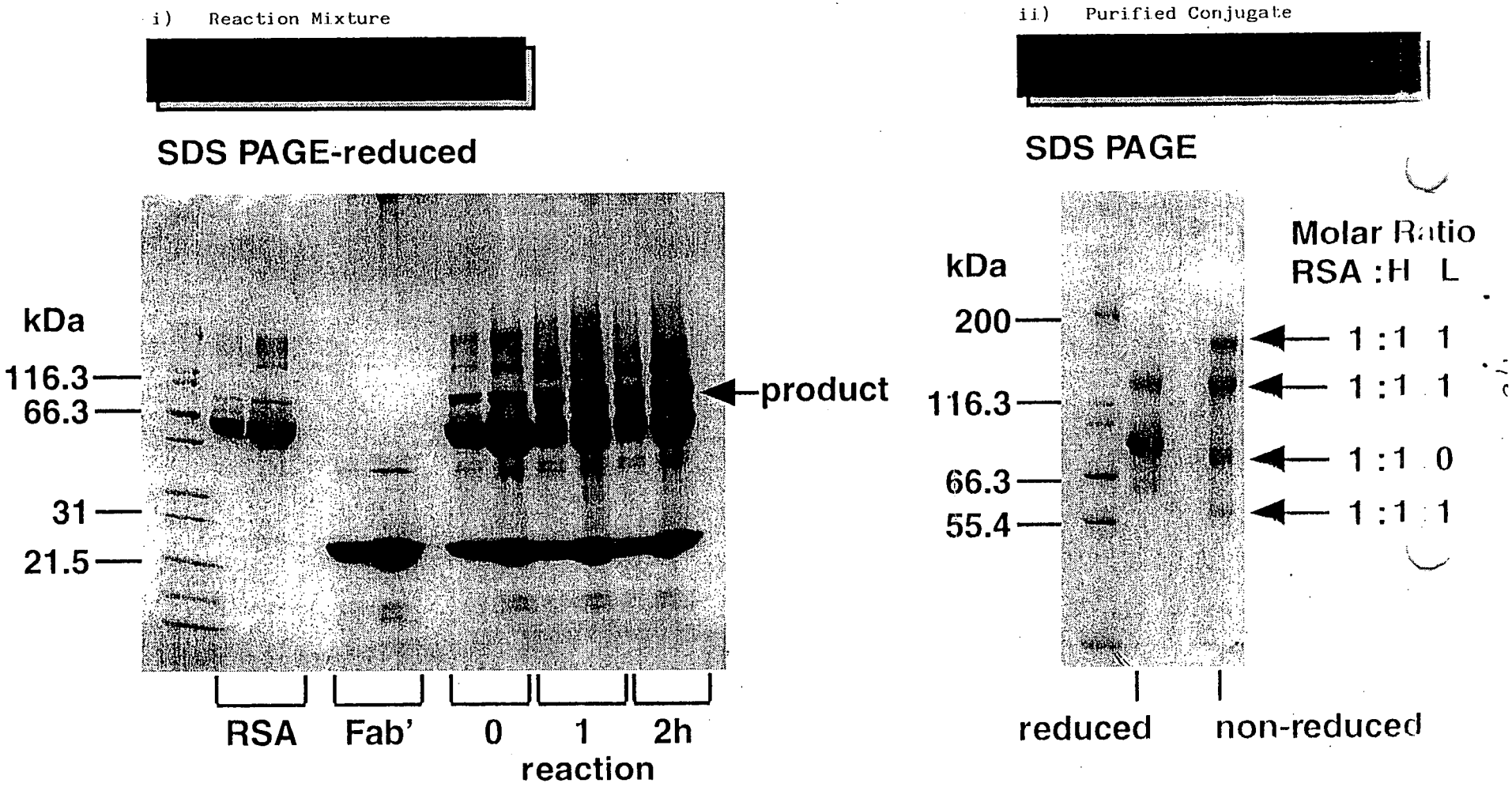


Fig. 1

RSA - hTNF40 Fab' conjugate: analysis

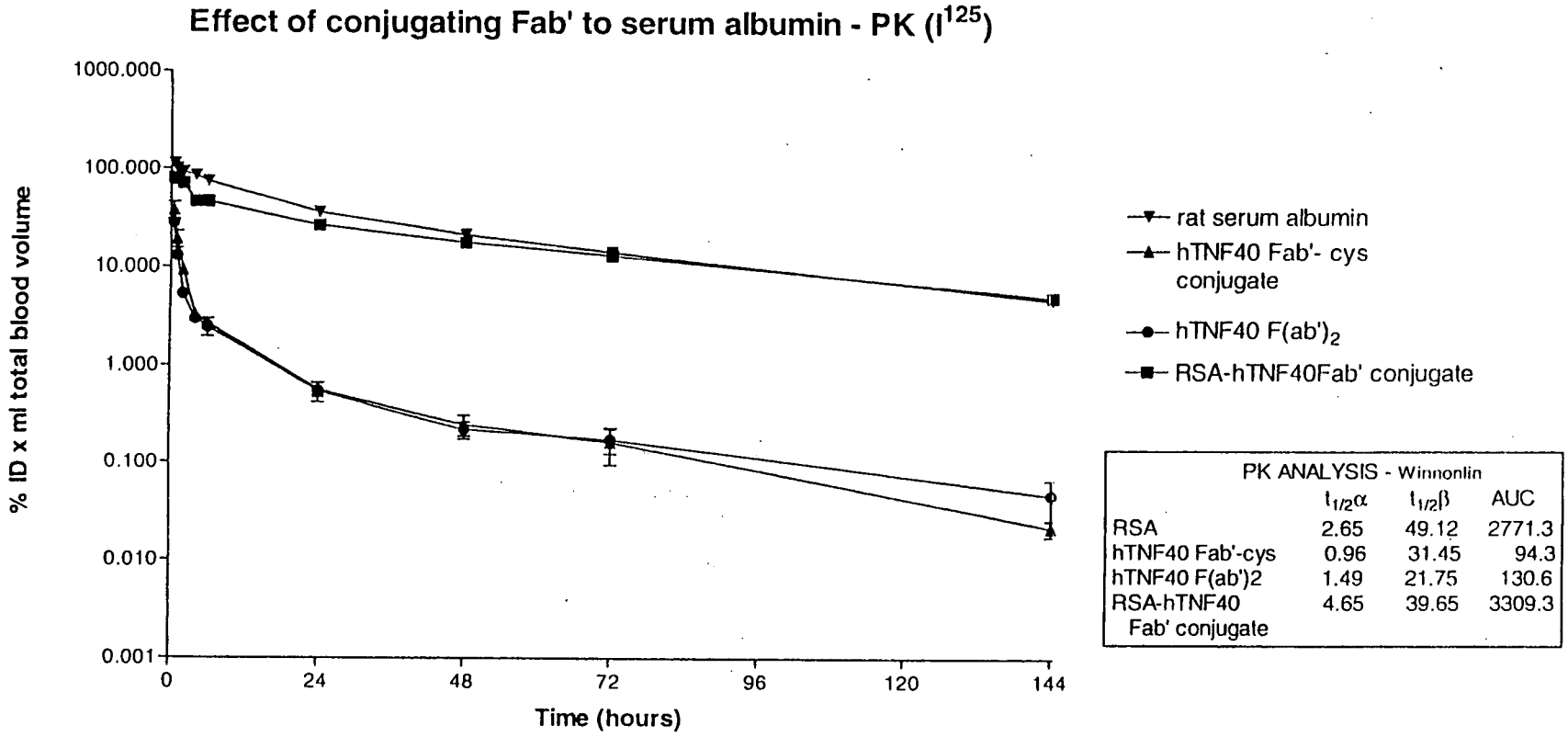
(i) By N-terminal sequencing.

	Approx. Mr app.	Molar Ratio, RSA: H chain: L chain
Whole prep	--	1 : 1 : 1
PAGE, reduced	90 kDa	1 : 1 : 0
PAGE, non-reduced	60	1 : 1 : 1
	80	1 : 1 : 0
	120	1 : 1 : 1
	>170	1 : 1 : 1

(ii) By Biacore: binding of TNF

	kass, 10^5 $M^{-1}s^{-1}$	kdiss, 10^{-4} s^{-1}	KD, 10^{-10} M
RSA - hTNF40 Fab'conj.	3.88	1.65	4.25
hTNF40 IgG	3.63	1.41	3.88
hTNF40 Fab'	2.79	0.56	2.01

Fig. 2



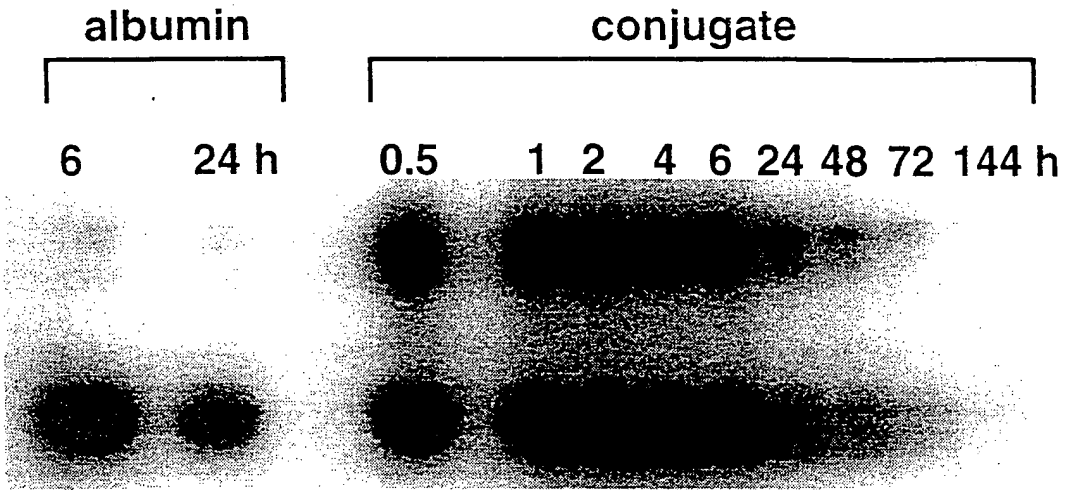
PK ANALYSIS - Winnonlin

	$t_{1/2}\alpha$	$t_{1/2}\beta$	AUC
RSA	2.65	49.12	2771.3
hTNF40 Fab'-cys	0.96	31.45	94.3
hTNF40 F(ab')2	1.49	21.75	130.6
RSA-hTNF40 Fab' conjugate	4.65	39.65	3309.3

Fig. 3

3/6

Scanned PAGE of ^{125}I-RSA or RSA-Fab' conjugate in rat plasma *in vivo*



SDS PAGE: non-reduced

Changed in abundance of conjugate 120 and 60 kDa species with time *in vivo*

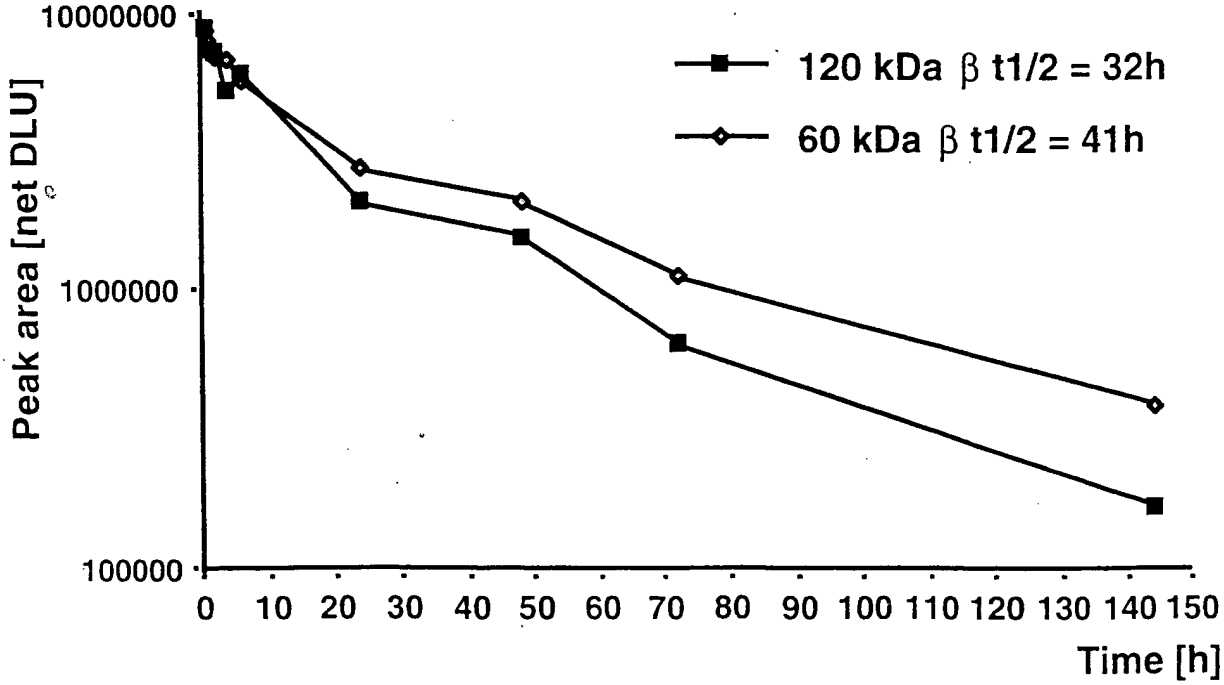


Fig. 4

Effect of conjugating Fab' to serum albumin - PK(ELISA)

PK ANALYSIS- Winnonlin		$t_{1/2}\alpha$	$t_{1/2}\beta$	AUC
hTNF40 Fab'-cys	4.6 0		n/a	3525.7
	2.21		n/a	3359.9
RSA-hTNF40	10.18		38.0	990458.9
Fab' conjugate	0.11		15.71	869156.1

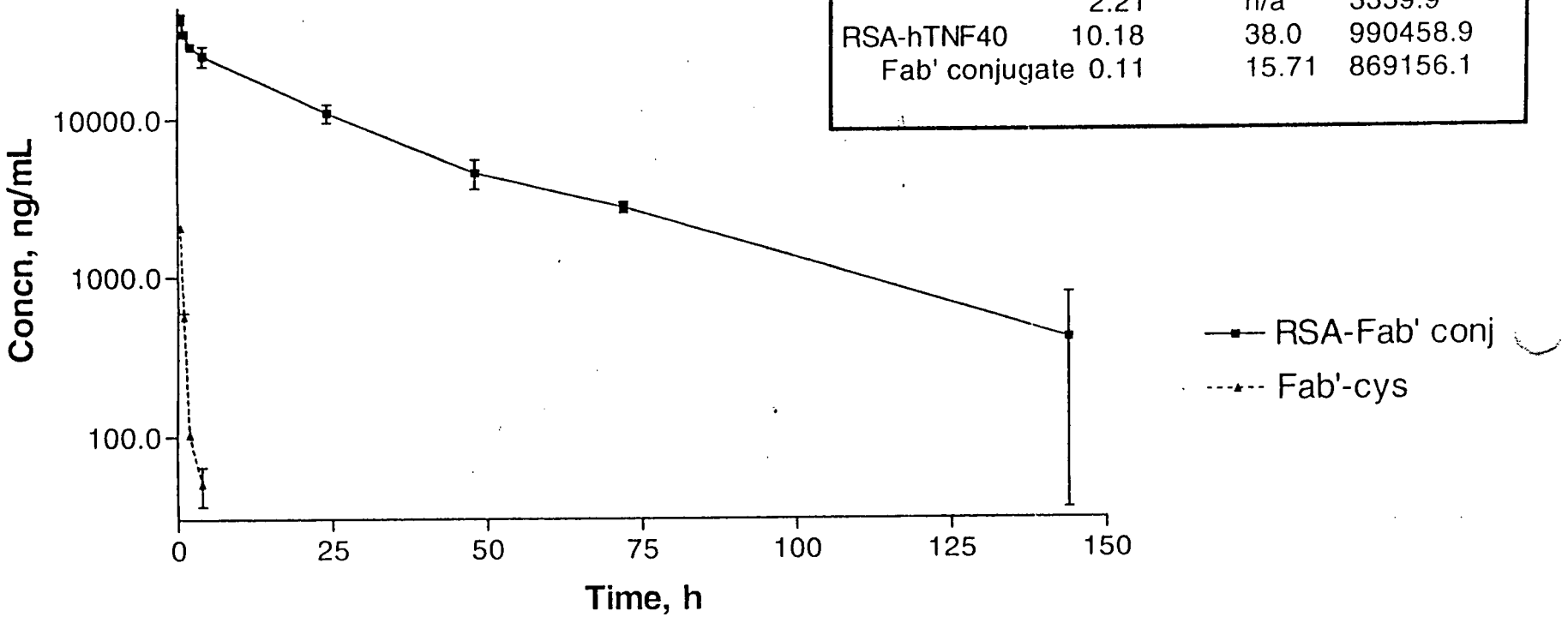


Fig. 5

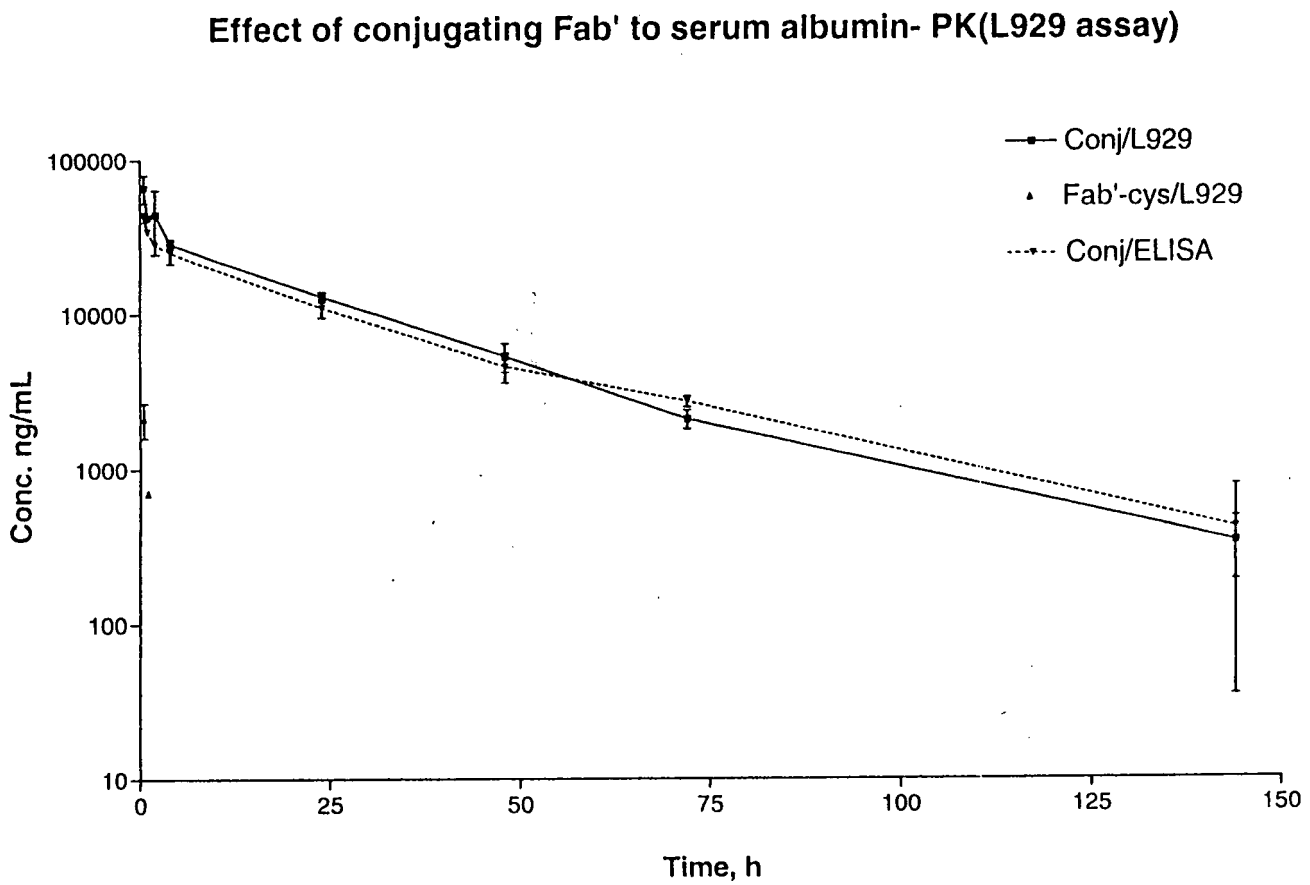

Effect of conjugating Fab' to serum albumin- PK(L929 assay)
Conj/L929
Fab'-cys/L929
Conj/ELISA
Conc. ng/mL
100000
10000
1000
100
10
0
25
50
75
100
125
150
Time, h

Fig. 6

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AGENT: Carpmaels & Ransford

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